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Origin of CD57$^+$ T cells which increase at tumour sites in patients with colorectal cancer

T. OKADA, T. IIAI, Y. KAWACHI, T. MORODA, Y. TAKII, K. HATAKEYAMA & T. ABO*
*First Department of Surgery and *Department of Immunology, Niigata University School of Medicine, Niigata, Japan*

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SUMMARY

Human T cells carrying natural killer (NK) markers, CD57 or CD56 antigens, appear to be distinguishable from other T cell subsets in terms of their granular lymphocyte morphology and their numerical increase in patients with AIDS and in recipients of bone marrow transplantation. At the begining of this study, we observed that CD57$^+$ T cells as well as CD56$^+$ T cells were abundant at tumour sites in many patients with colorectal cancer. Since all these findings for CD57$^+$ T cells are quite similar to those of extrathymic T cells seen in mice, we investigated how CD57$^+$ T cells are distributed to various immune organs in humans. They were found to be present mainly in the bone marrow and liver, but to be completely absent in the thymus. Similar to the case of extrathymic T cells in mice, they were observed to consist of double-negative CD4$^-$8$^-$ subsets as well as single-positive subsets (preponderance of CD8$^+$ cells), and to contain a considerable proportion of $\gamma\delta$ T cells. These features are striking when compared with those of CD57$^-$ T cells, which are characterized by an abundance of CD4$^+$ subsets and $\alpha\beta$ T cells. Not only at tumour sites but also in the peripheral blood, some patients with colorectal cancer displayed elevated levels of CD57$^+$ cells. These results suggest that CD57$^+$ T cells may be of extrathymic origin, possibly originating in the bone marrow and liver, and may be associated with tumour immunity, similar to another extrathymic population of CD56$^+$ T cells in humans.

Keywords CD57$^+$ T cells tumour-infiltrating lymphocytes bone marrow liver colorectal cancer

INTRODUCTION

We previously demonstrated that CD56$^+$ T cells were present among tumour-infiltrating lymphocytes (TIL) and significantly increased proportionately among mononuclear cells (MNC) of the peripheral blood in patients with colorectal cancer [1]. CD56$^+$ T cells in these patients as well as in healthy individuals were found to have unique properties distinguishable from those of usual CD56$^-$ T cells. Namely, they had the morphology of large granular lymphocytes (LGL) similar to natural killer (NK) cells, contained double-negative (DN) CD4$^-$8$^-$ cells, and comprised $\gamma\delta$ T cells as well as $\alpha\beta$ T cells. More importantly, the liver was the immune organ with the greatest abundance of CD56$^+$ T cells. All of these properties coincide with those of extrathymic T cells which have been intensively characterized in mice [2–6]. In the case of mice, such extrathymic T cells are identified as intermediate T cell receptor (TCR) cells or NK1.1$^+$ T cells. In these respects, we postulated that CD56$^+$ T cells might be a counterpart of extrathymic T cells in humans [1]. The only difference is that CD56$^+$ T cells in humans do not have intermediate levels of TCR (or CD3). Extrathymic T cells seen in mouse liver, but not in mouse intestine, carry intermediate levels of TCR and are therefore termed intermediate TCR cells.

In the course of studies on CD56$^+$ T cells in cancer patients, we have observed that CD57$^+$ T cells are much more abundant than CD56$^+$ T cells among TIL and MNC of peripheral blood in these patients. It was reported in an earlier study that CD57$^+$ T cells also had the morphology of LGL [7]. In this study, we further characterized CD57$^+$ T cells in healthy individuals and cancer patients. It was demonstrated that CD57$^+$ T cells also had unique properties distinguishable from those of usual CD57$^-$ T cells. One of the most striking pieces of evidence was that CD57$^+$ T cells existed most abundantly in the bone marrow. On the other hand, CD56$^+$ T cells are abundant in the liver. Even in mice, the bone marrow as well as the liver are known to be the sites for extrathymic T cell differentiation [8,9]. Taking into account these facts together with recent reports that CD57$^+$ T cells increased in patients with AIDS [10–12] and in those subjected to bone marrow transplantation [13–15],

Correspondence: Toru Abo, Department of Immunology, Niigata University School of Medicine, Asahimachi-dori 1, Niigata 951, Japan.

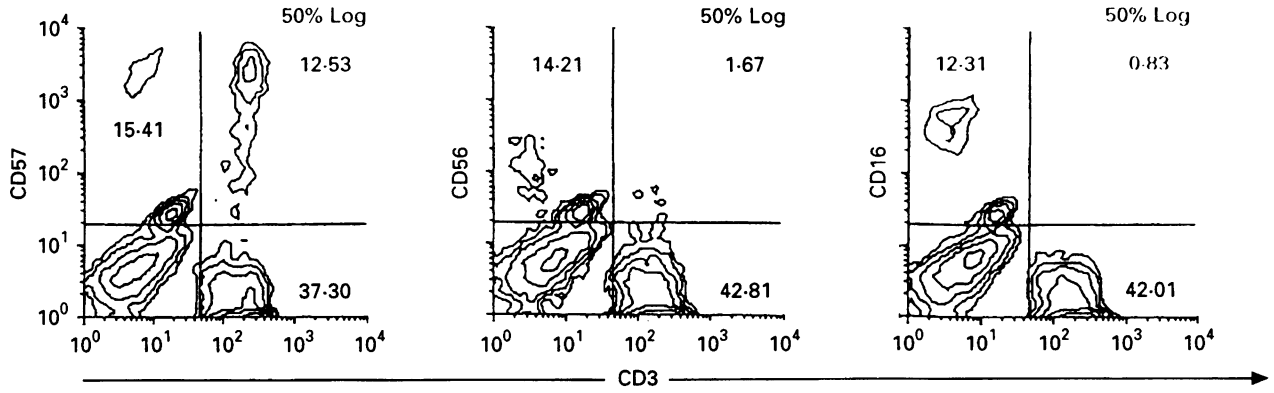

Fig. 1. Identification of CD57$^+$ T cells in tumour-infiltrating lymphocytes (TIL) isolated from a patient with colorectal cancer. Two-colour staining of TIL for CD3 and each natural killer (NK) marker indicated in the figure was performed. CD57$^+$ T cells were abundant in this TIL preparation, whereas CD56$^+$ T and CD16$^+$ T cells were very few. CD3$^-$CD57$^+$, CD3$^-$CD56$^+$, and CD3$^-$CD16$^+$ cells of NK populations were also present. Some false-positive cells, which might be cell debris, are present in the NK fraction. Numbers in the figure indicate the percentages of fluorescence-positive cells in the corresponding areas.

it is conceivable that CD57$^+$ T cells might be another population of extrathymic T cells in humans.

PATIENTS AND METHODS

Patients

Forty-eight patients (29 men and 19 women) with colorectal cancer, ranging in age from 24 to 81 years, were included in this study. Five patients were at stage A, 11 patients were at stage B, 10 patients were at stage C, and seven patients were at stage D according to Duke's classification of colorectal cancer [1]. The others with colorectal cancer were 14 patients with metachronous liver metastasis, and one patient with paraaortic lymph node recurrence.

Age-matched controls (n = 18) were four normal healthy subjects (three men and one woman) and 14 patients with benign diseases (six men and eight women), ranging in age from 21 to 86 years. Benign controls included patients with benign thyroid tumours and cholecystolithiasis.

Cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood [1]. MNC were obtained from the interface by Ficoll–Isopaque gradient (1·077) centrifugation.

Liver, primary tumour, thymus and lymph nodes were obtained from resected specimens of controls or patients with colorectal cancer who underwent colectomy or hepatectomy. Bone marrow was obtained from patients with leukaemia or from the benign controls, and spleen from patients with gastric cancer or from the benign controls. Specimens were used when apparent signs of diseases in the corresponding sites were not observed. All materials were used with the permission of the patients and the ethics committee of Niigata University.

MNC of the bone marrow were collected by Ficoll–Isopaque centrifugation, similar to the case of peripheral blood. To prepare MNC from the liver, thymus and lymph nodes, the samples were cut into small pieces with scissors. In the case of tumour tissues, the samples were similarly processed and then treated with collagenase (1 mg/ml), DNAse (0·1 mg/ml) and hyaluronidase (0·1 mg/ml) (Sigma Chemical Co., St Louis, MO) at 37°C for 60 min [1]. Treated samples of the liver, thymus, spleen, lymph nodes, and primary tumour were pressed through 200 G stainless steel mesh and suspended in RPMI 1640 medium supplemented with 2% heat-inactivated newborn calf serum. The suspension was centrifuged by the Ficoll–Isopaque method. The MNC were collected from the interface of Ficoll–Isopaque gradient centrifugation and suspended in PBS.

Phenotyping of cells by flow cytometry

MNC derived from the peripheral blood, liver, spleen, lymph nodes and tumour were incubated with FITC-, PE-, Per-CP, or biotin-conjugated MoAbs [1]. Such MoAbs included CD3 (NU-T3; Nichirei, Tokyo, Japan), CD4 (Leu-3a), CD8 (NU-Ts/c; Nichirei), CD56 (Leu-19), CD57 (Leu-7), CD16 (Leu-11), TCR-$\alpha\beta$ (TCR-1) (Becton Dickinson, Mountain View, CA), and TCR-$\gamma\delta$ (TCRδ1) (T Cell Sciences, Cambridge, MA). Two- or three-colour flow cytometric analysis was performed by using a fluorescence-activated cell analyser (FACScan; Becton Dickinson). Ten thousand cells in the two-colour staining and 30 $\times$ 10^3 cells in the three-colour staining were analysed, respectively.

Statistical analysis

Statistical analysis of the parameters in the data from the peripheral blood lymphocytes was performed by Student's t-test. In other cases, the Wilcoxon test was employed for such analysis. Differences were taken to be significant when P was $<0·05$.

RESULTS

Abundance of CD57$^+$ T cells in TIL of patients with colorectal cancer

Although we previously reported that CD56$^+$ T cells were abundant in TIL of patients with colorectal cancer [1], we noticed that CD57$^+$ T cells were more abundant than CD56$^+$ T cells in TIL in some cases. A typical case is represented in Fig. 1. To identify T cells coexpressing NK

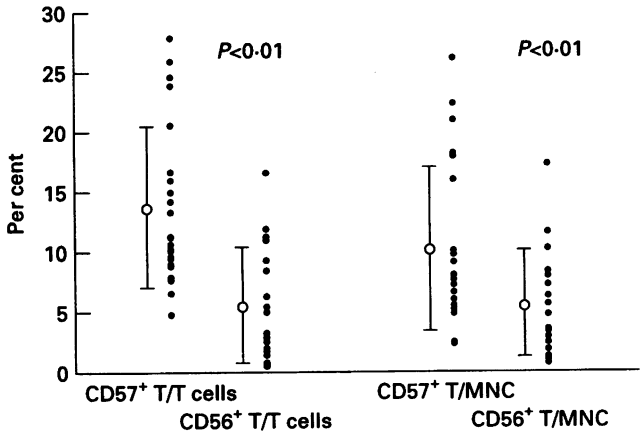

Fig. 2. Abundance of CD57$^+$ T cells in tumour-infiltrating lymphocytes (TIL) of patients with colorectal cancer. The percentages of CD57$^+$ T cells and CD56$^+$ T cells are plotted among the total T cell population or among mononuclear cells (MNC). CD57$^+$ T cells infiltrated the tumour sites more frequently than did CD56$^+$ T cells in either case (n = 22, P < 0·01).

marker, two-colour staining for CD3 and each NK marker (CD57, CD56 and CD16) was carried out. It was observed that one-quarter of the T cells in TIL of this patient with colorectal cancer were CD57$^+$ T cells. This level was greater than that of CD3$^-$ NK cells expressing CD57, CD56 or CD16 antigens in this TIL preparation. Because of cell debris in the preparation, some false-positive cells were seen in the NK fractions. CD56$^+$ T cells as well as CD16$^+$ T cells were quite few in number.

A similar analysis was then performed in many cases of colorectal cancer (n = 22) (Fig. 2). The percentages of CD57$^+$ T cells and CD56$^+$ T cells in TIL are presented on the basis of total T cells or total MNC. In both presentations, CD57$^+$ T cells can be seen to be much more abundant than CD56$^+$ T cells in TIL, although some patients also showed a considerable proportion of CD56$^+$ T cells.

The bone marrow is the organ with the greatest abundance of CD57$^+$ T cells

Originally, CD57$^+$ T cells were found to exist as a minor population in the peripheral blood of healthy individuals [7]. In this experiment, we examined how CD57$^+$ T cells are distributed among various immune organs, in order to help

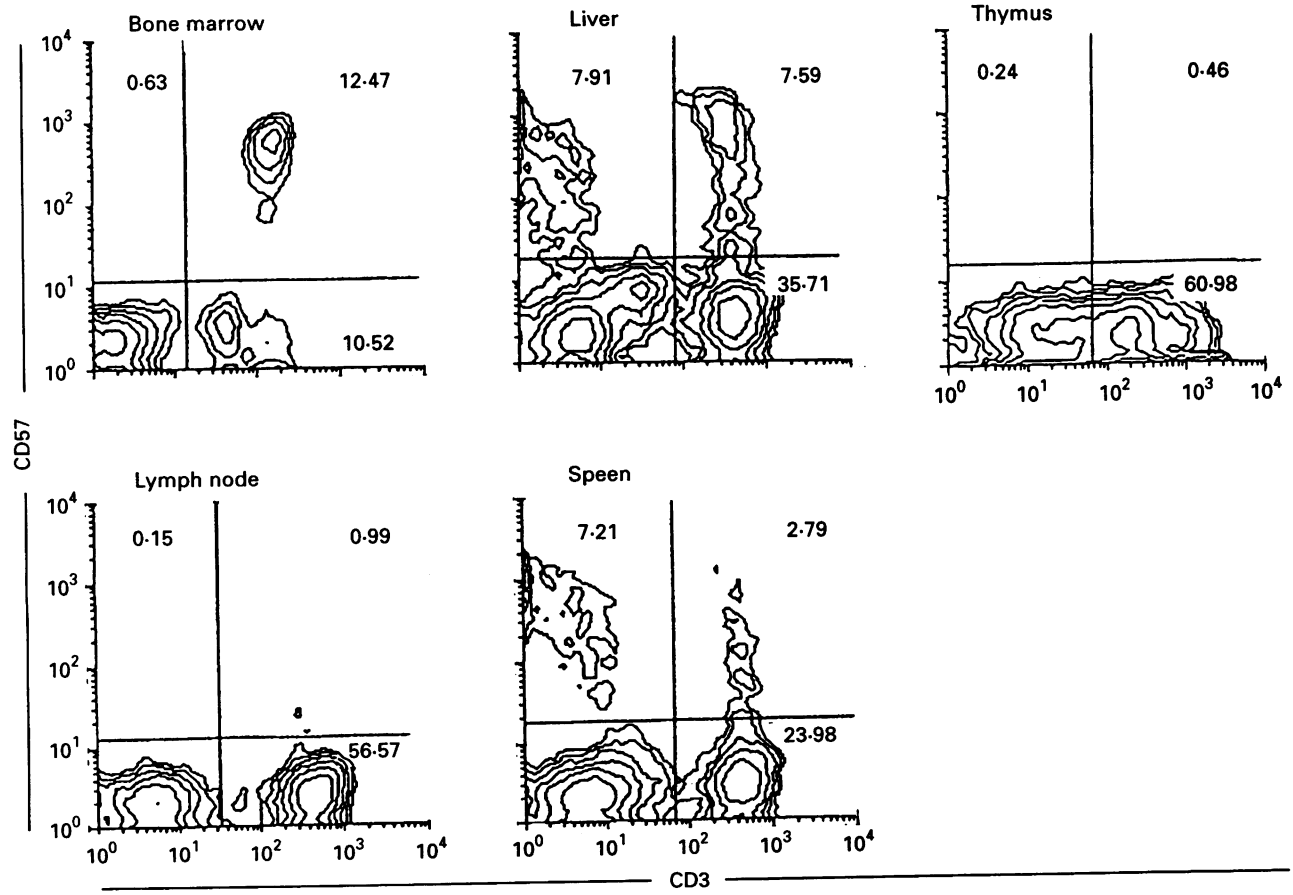

Fig. 3. CD57$^+$ T cells are present in not only the liver but also the bone marrow. Mononuclear cells (MNC) were isolated from various patients, including cancer patients, leukaemia patients and others. A typical pattern is represented here, in which the bone marrow and liver were derived from one patient and other organs were from another patient. Two-colour staining for CD3 and CD57 was performed. Numbers indicate the percentages of fluorescence-positive cells in the corresponding areas. Results were confirmed by repeated experiments as shown in Fig. 4.

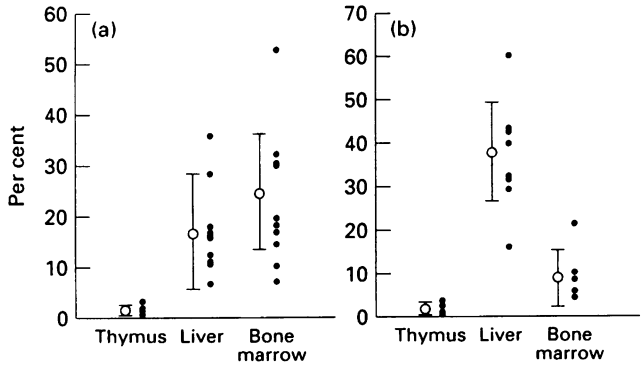

Fig. 4. Distribution of CD57$^+$ T and CD56$^+$ T cells in the thymus, liver, and bone marrow. (a) CD57$^+$ T cells. (b) CD56$^+$ T cells. Mononuclear cells (MNC) were isolated from the thymus (n = 5), liver (n = 10) and bone marrow (n = 10) in patients undergoing surgical operation. The percentages of CD57$^+$ T and CD56$^+$ T cells were estimated by two-colour staining for CD3 and CD57 (or CD56). CD57$^+$ T cells were abundant both in liver and in bone marrow, whereas CD56$^+$ T cells were abundant only in the liver.

determine their origin (Fig. 3). Since it was impossible to obtain all materials from the same healthy individual, MNC were isolated from corresponding organs of several cases without apparent abnormalities. In one typical case, CD57$^+$ T cells were observed to be most abundant in the bone marrow. More than 50% of all T cells were CD57$^+$ T cells in this case. On the other hand, the proportions of CD57$^+$ T cells in other organs were very low, although a considerable proportion of CD57$^+$ T cells were seen in the liver and spleen. It should be noted that CD57$^+$ T cells were not present at all in the thymus, nor in the lymph nodes.

To confirm the above results, the proportions of CD57$^+$ T cells among total T cells were further examined in the thymus, liver, and bone marrow in many cases (Fig. 4a). Those of CD56$^+$ T cells were examined in parallel (Fig. 4b). It was clearly demonstrated that CD57$^+$ T cells were abundant both in the bone marrow and in the liver. In contrast, CD56$^+$ T cells were abundant only in the liver. Neither CD56$^+$ T cells nor CD57$^+$ T cells were present in the thymus.

The composition of DN CD4$^-$8$^-$, CD4$^+$, CD8$^+$, and γδ T cells in the population of CD57$^+$ T and CD56$^+$ T cells

In a previous study [7] we reported that CD57$^+$ T cells were unique in terms of morphology, LGL. In this study, we examined their phenotypic characteristics, especially how they comprise CD4$^-$8$^-$ cells, γδ T cells and others (Fig. 5). The materials in Fig. 5 were obtained from one cancer patient undergoing surgical operation. For this purpose, three-colour staining for CD3, CD57 and others (indicated in the figure) was performed. The gating analysis of CD57$^+$ T cells revealed that CD4$^-$8$^-$ cells were quite abundant in all organs tested, especially in the bone marrow (60·0%). The proportion of CD8$^+$ cells was much more predominant than that of CD4$^+$ cells. Moreover, a significant proportion of γδ T cells was also detected in this population.

To determine further the composition of DN, CD4$^+$, and CD8$^+$ cells in CD57$^+$ T and CD56$^+$ T cells, three-colour staining for CD57 (or CD56), CD4, and CD8 was carried out in various cases (Fig. 6a). In these experiments, MNC in the peripheral blood of healthy individuals were used (n = 8). In

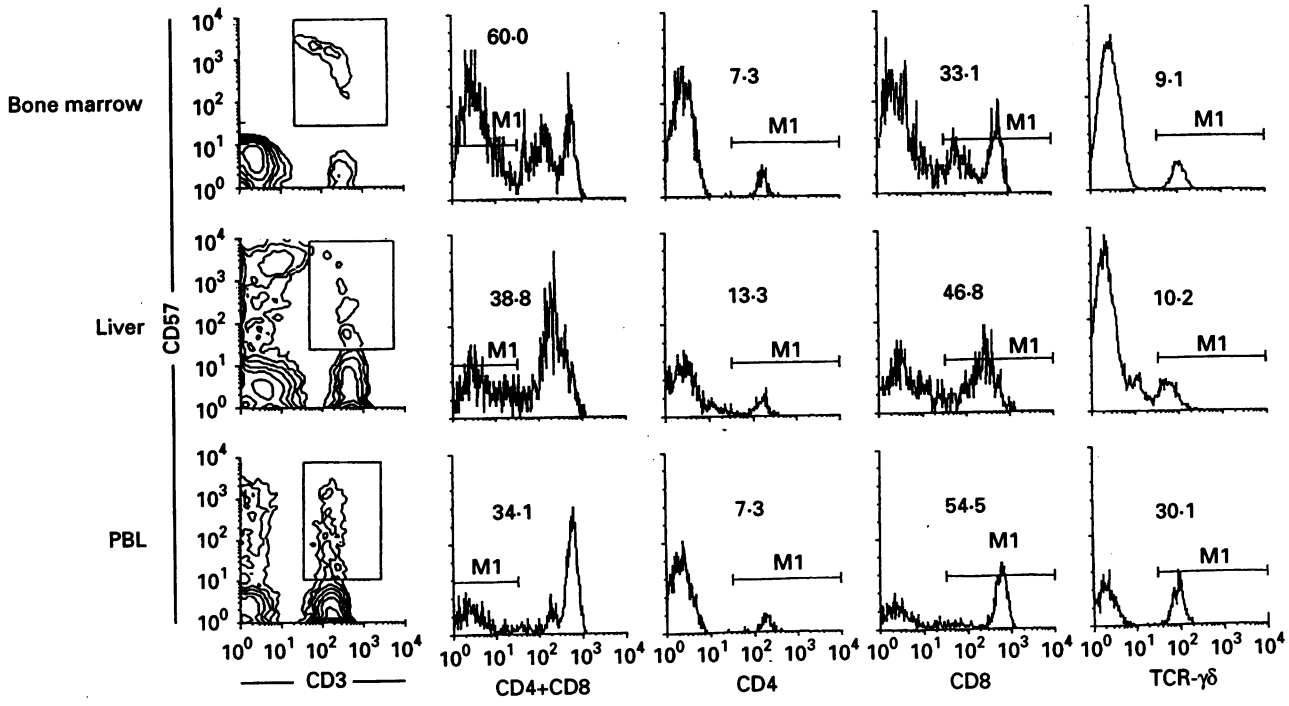

Fig. 5. Preponderance of double-negative (DN) CD4$^-$8$^-$ cells, CD8$^+$ cells and γδ T cells among CD57$^+$ T cells in the bone marrow, liver and blood. A typical pattern seen in CD57$^+$ T cells was represented in the bone marrow, liver and peripheral blood. Three-colour staining for CD3, CD57 and others (indicated in the figure) was performed and the gating analysis for CD57$^+$ T cells (encompassed by squares) was done to identify their phenotypic features. Numbers represent the fluorescence-positive cells for corresponding area.

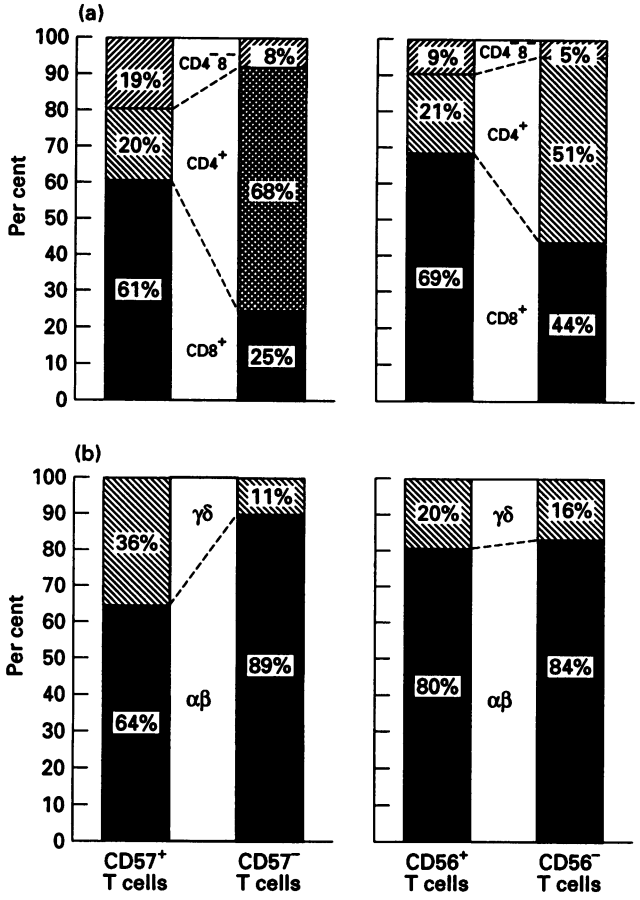

Fig. 6. Further phenotypic characterization of CD57$^+$ T and CD56$^+$ T cells. (a) Composition of double-negative (DN) CD4$^-$8$^-$, CD4$^+$, and CD8$^+$ cells, (b) Composition of $\gamma\delta$ T and $\alpha\beta$ T cells. Mononuclear cells (MNC) were isolated from the peripheral blood of healthy individuals ($n = 8$). For simplification of the figure, the squares are divided according the mean value of each subset. DN CD4$^-$8$^-$, CD8$^+$, and $\gamma\delta$ T cells were predominant in CD57$^+$ T cells.

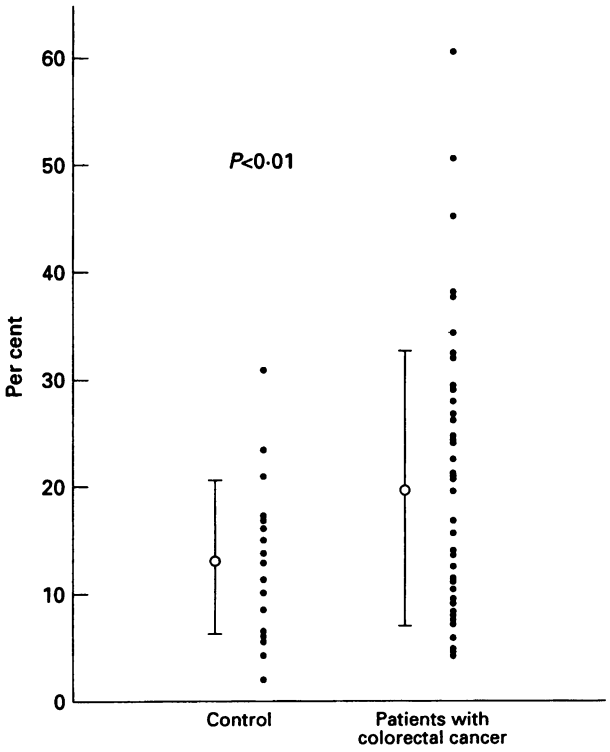

Fig. 7. An increase in the proportion of CD57$^+$ T cells is seen even in blood mononuclear cells (MNC) of patients with colorectal cancer. The values of cancer patients ($n = 48$) as well as of age-matched healthy controls ($n = 18$) are plotted. These cancer patients were matched with those in Figs 1 and 2. The values of cancer patients were significantly higher than those of controls ($P < 0{\cdot}01$).

the case of CD57$^+$ T cells, CD8$^+$ cells were predominant, while CD4$^+$ cells were very few. DN CD4$^-$8$^-$ cells were also abundant in CD57$^+$ T cells. All these features were striking compared with those of CD57$^-$ T cells, because CD4$^+$ cells were overwhelmingly abundant in this population. CD56$^+$ T cells had a composition pattern similar to that of CD57$^+$ T cells, although the deviation of composition from their negative subsets was not so striking as in the case of CD57$^+$ T cells.

By using the same materials as in Fig. 6a, the composition of $\alpha\beta$ T and $\gamma\delta$ T cells in CD57$^+$ T and CD57$^-$ T cells was examined (Fig. 6b). It was clearly demonstrated that CD57$^+$ T cells contained a higher proportion of $\gamma\delta$ T cells than their negative subsets. This was more striking than in the case of CD56$^+$ T cells.

Increase of CD57$^+$ T cells in the peripheral blood of patients with colorectal cancer

As shown in Fig. 1, TIL isolated from patients with colorectal cancer contained a high level of CD57$^+$ T cells. In this experiment, we investigated whether such abnormally high levels of CD57$^+$ T cells were seen in the peripheral blood of patients with colorectal cancer (Fig. 7). Although many patients displayed proportions of CD57$^+$ T cells within a normal range, some patients displayed apparently higher levels of CD57$^+$ T cells ($P < 0{\cdot}01$).

We then classified patients with colorectal cancer into four groups, A–D, and an additional group having metachronous

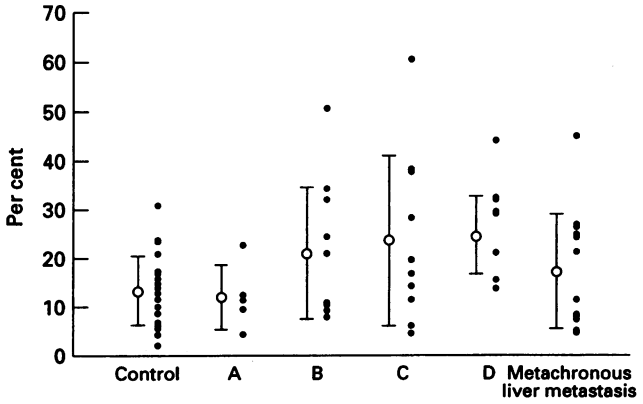

Fig. 8. Relationships between the levels of CD57$^+$ T cells and the stage of malignancy (Duke's classification) in cancer patients. The values of CD57$^+$ T cells in blood mononuclear cells (MNC) of patients with colorectal cancer are plotted. The levels of CD57$^+$ T cells in the blood tended to increase as a function of the severity of malignancy.

liver metastasis (Fig. 8). Among these groups, the levels of $CD57^+$ T cells in the peripheral blood tended to increase as a function of the severity of malignancy, except the last group. Because of considerable variations in the levels of $CD57^+$ T cells among the stages of malignancy, the statistical significance of the increase of $CD57^+$ T cells with severity was not produced by applied analyses.

DISCUSSION

Since the identification of NK markers, CD57 [16] and CD56 [17], by MoAbs, many investigators have noticed that there is a small population of T cells which expresses these antigens on the surface [7,18,19]. CD56 antigen is now known to be neural cell adhesion molecule-1 (NCAM-1) [20], while CD57 antigen is a sulphated carbohydrate determinant on glycoprotein of lymphoid cells or on myelin-associated glycoprotein of neural cells [21]. $CD56^+$ T and $CD57^+$ T cells have the morphology of LGL [7,18], similar to NK cells having the phenotypes of $CD3^-CD56^+$ and $CD3^-CD57^+$. In the present study, we demonstrated that $CD57^+$ T cells were prominent in TIL isolated from patients with colorectal cancer. Although we previously reported that $CD56^+$ T cells were present among TIL [1], the levels of $CD57^+$ T cells among TIL were found to be higher than those of $CD56^+$ T cells in this study.

To determine the origin of $CD57^+$ T cells, we first examined which immune organs contained the largest proportion of $CD57^+$ T cells. It was demonstrated that $CD57^+$ T cells were abundant both in the bone marrow and liver. This was a very interesting result compared with the localization of $CD56^+$ T cells. Namely, $CD56^+$ T cells were found to be abundant only in the liver. The thymus did not contain $CD57^+$ T cells or $CD56^+$ T cells. It is possible that T cells which maturate in the thymus acquire CD57 or CD56 antigens after migrating to the corresponding organs. However, it is also possible that these $CD57^+$ T and $CD56^+$ T cells are generated extrathymically in each organ. Concerning many properties such as the morphology of LGL and the partial composition of DN $CD4^-8^-$ cells and $\gamma\delta$ T cells seen in these populations, it is speculated that they might be counterparts of extrathymic T cells in humans. The properties of extrathymic T cells as primitive T cells in mice have been intensively characterized [2–6]. We postulate that extrathymic T cells might be developed earlier than thymus-derived T cells in phylogeny.

Several investigators have reported that $CD57^+$ cells were one of the major infiltrating lymphocytes into the tumour sites, especially in patients with gastric carcinoma [22–24]. However, they did not examine whether or not such $CD57^+$ cells express CD3 (or TCR). In other words, they dealt with these cells as NK cells rather than extrathymic T cells.

Concerning mice, many investigators have reported that extrathymic T cells exist at multiple sites of the body, including the liver [2–6], intestine [25,26], bone marrow [8,9], omentum [27,28], skin [29,30], and uterus [31], raising the possibility that these cells are generated in various organs. If this is the case, even in humans, $CD57^+$ T cells may be generated in both bone marrow and liver, while $CD56^+$ T cells may be generated in the liver. We recently observed that the initial recovery of T cells in irradiated mice (9 Gy) commenced in intermediate TCR cells (i.e. extrathymic T cells in mice) after bone marrow transplantation. Even in the case of humans, almost all T cells which are initially seen in the bone marrow and other peripheral organs after bone marrow transplantation are reported to be $CD57^+$ T cells [13–15,32]. It is therefore conceivable that $CD57^+$ T cells are a major population of extrathymic T cells generated in the bone marrow.

When investigators focused their attention on $CD57^+$ T cells in various diseases, such cells were often identified as $CD8^+$ $CD57^+$ cells [12,15,33,34]. Data from the present study confirm that observation, because most $CD57^+$ T cells were $CD8^+$. However, it should be remembered that they also included $CD4^+$ cells and DN $CD4^-8^-$ cells. This is true even in the case of $CD56^+$ T cells [1]. In mouse studies, we found DN $CD4^-8^-$ cells to be confined to intermediate TCR cells of extrathymic origin [2–6]. This was true even in the case of humans, because DN $CD4^-8^-$ cells are confined to $CD56^+$ T or $CD57^+$ T cells [35,36]. Moreover, most $CD8^+$ cells among intermediate TCR cells in mice have the $\alpha\alpha$ homodimer of CD8 antigens [5]. Similarly, it has been reported that $CD57^+$ T cells in humans also have the $\alpha\alpha$ homodimer of CD8 antigens [37]. Namely, $CD57^+$ (or $CD56^+$) T cells in humans and intermediate TCR cells in mice share common properties.

When transgenic mice with TL antigen (i.e. monomorphic MNC antigen) were examined, most expanding cells were found to be intermediate TCR cells with DN $CD4^-8^-$ phenotype [38]. On the other hand, in mice which suffered from autoimmune-like chronic graft-*versus*-host disease (GVHD) due to the injection of T cells with MHC class II disparity, expansion of intermediate TCR cells with $CD4^+$ phenotype was observed [39]. In this regard, minor subsets of $CD4^+$ or DN $CD4^-8^-$ among $CD57^+$ T cells can not be neglected, especially in diseased conditions. In other words, there is some positive selection even in the primitive, extrathymic pathways of T cell differentiation.

In mouse studies, we demonstrated that more than 50% of T cells which infiltrated tumour sites were intermediate TCR cells, including their $CD8^+$ and DN $CD4^-8^-$ subsets [40]. Even in the case of humans, one-quarter of TIL were either $CD57^+$ T or $CD56^+$ T cells. Since it is known that extrathymic T cells cannot completely eliminate self-reactive forbidden clones [3], it is presumed that such self-reactive clones might be beneficial against malignant cells. Additional data have indicated that intermediate TCR cells are eventually associated with cancer immunity by their cytotoxic function [41].

$CD57^+$ T cells have received much attention in studies on AIDS [10–12]. It was reported that T cells remaining in patients at the end stage of AIDS are $CD8^+$ $CD57^+$ T cells [10]. Even in a mouse AIDS model (MAIDS), the most resistant T cells against retroviral infections are intermediate TCR cells of extrathymic origin (H. Watanabe *et al.*, manuscript submitted). Similarly, the most radio-resistant T cells were found to be intermediate TCR cells in a mouse study [42]. Against either retroviral infections or irradiation, primitive T cells, $CD57^+$ T (or $CD56^+$ T) cells in humans and intermediate TCR cells in mice, seem to be more pertinacious than thymus-derived T cells. Therefore, the function of primitive T cells in the above described conditions, including malignancy, AIDS, and even autoimmune diseases, is deserving of attention. $CD57^+$ T cells are known to increase in several types of autoimmune diseases [43,44]. It is therefore conceivable that $CD57^+$ T cells have important immunological functions in many diseases.

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